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(54) GENETICALLY MODIFIED CELLS FOR RESONANCE ENERGY TRANSFER ASSAY WITH SYNAPTOBREVIN SUBSTRATE MOIETY

GENETISCH MODIFIZIERTE ZELLEN FÜR RESONANZENERGIE-TRANSFERASSAY MIT EINEM SYNAPTOBREVIN-FRAGMENT ALS SUBSTRAT

CELLULES GENETIQUEMENT MODIFIÉES POUR ANALYSE PAR TRANSFERT D'ÉNERGIE DE RÉSONANCE UTILISANT, COMME SUBSTRAT, UN FRAGMENT DE SYNAPTOBRÉVINE

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Description

Field of The Invention

[0001] The field of the invention is Forster resonance energy transfer (FRET) assays for protease activity, especially protease assays for *Botulinum* neurotoxins BoNTs that cleave synapto brevin.

Background of the Invention

[0002] *Botulinum* neurotoxins (BoNTs) are extremely toxic proteins and can be classified into distinct subgroups based, *inter alia,* on peptide sequence and/or substrate specificity. All of the naturally occurring BoNTs (BoNT/A-G) are composed of a heavy chain that mediates toxin entry into a target cell and a light chain with zinc-dependent protease activity that hydrolyzes selected SNARE proteins that mediate fusion of neurotransmitter vesicles to the membrane that forms part of the synaptic cleft.

[0003] For example, the light chain of BoNT/A hydrolyzes with high specificity SNAP-25, which is required for vesicle-mediated exocytosis of acetylcholine into the synaptic cleft. Known assays for such hydrolytic activity include those described in our copending International application (WO 2009/035476). Here, a fluorophore and a quencher are covalently linked to the respective ends of a peptide sequence that includes, for example, the SNAP-25 sequence. Cleavage by BoNT/A (or other BoNTs with a substrate specificity towards SNAP-25) will result in physical separation of the cleavage products and so reduce fluorescence quenching, which can then be quantified. Among other choices, it is often preferred that such assay is performed as an *in vitro* solid-phase based assay.

[0004] While such assay is conceptually simple and can be used to readily determine BoNT/A, BoNT/C, or BoNT/E activity, such assay can not be simply modified to a cell-based assay for determination of BoNT/B, BoNT/D, BoNT/F, or BoNT/G activities by replacing the SNAP-25 motif with a SNARE domain as the SNARE domain includes a membrane spanning sub-domain that would place the N-terminal fluorophore into a vesicle lumen. In such case, only diffusion of the fluorescence signal would be observed as has been reported elsewhere (Dong et al. PNAS (2004), Vol. 101, No. 41, 14701-14706; or U.S. Pat. App. No. 2006/0134722).

[0005] Therefore, there is still a need for improved BoNT assays, and especially cell-based assays for BoNTs that cleave synaptobrevin.

Summary of the Invention

[0006] According to the present invention, genetically modified cells according to claims 1 and 8 and a kit according to claim 15 are provided for analyzing BoNT protease activity, and especially BoNT/B, BoNT/G, BoNT/D, and/or BoNT/F protease activity in a cell-based system using fluorescence resonance energy transfer. According to the present invention, the cells express one or more recombinant hybrid proteins together with at least one BoNT protease recognition and cleavage sequence, wherein the hybrid protein further comprises a transmembrane domain that is not cleavable by the BoNT protease and that directs the hybrid protein to an intracellular synaptic vesicle.

[0007] According to the present invention, it is provided a cell transfected with the nucleic acid presented herein, and it is generally preferred that the cell is stably transfected with the nucleic acid. Especially suitable cells include neuronal cells, neuroendocrine tumor cells, hybrid cells, and stem cells.

[0008] Most preferably, the transfected cell is a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, or a stem cell. It is further generally preferred that A includes a transmembrane domain from synaptobrevin, synaptophysin, synapsin I, synapsin II, and/or synapsin III, and/or that C includes at least two of a BoNT/B, a BoNT/G, a BoNT/D, and a BoNT/F protease recognition and cleavage sequence. While not limiting to the inventive subject matter, it is further preferred that a peptide linker is disposed between one or more of A and C, A and B, C and B, and C and D, and that the linker has a length of equal or less than 12 amino acids. Additionally, it is contemplated that the transfected cell may be contacted with a putative or known BoNT inhibitor prior to contacting the transfected cell with the BoNT protease. In especially preferred aspects, the transfected cell produces two hybrid proteins.

[0009] In exemplary embodiments, the hybrid protein having the structure of A-B-C-D has a sequence according to SEQ ID NO:2, the hybrid protein having the structure of A-C-B has a sequence according to SEQ ID NO:4, and the hybrid protein having the structure of A-C-B has a sequence according to SEQ ID NO:6.

[0010] According to one aspect of the present invention, the genetically modified cell comprises a recombinant nucleic acid including a sequence that encodes (I) a hybrid protein having a structure of A-B-C-D . According to a further aspect of the present invention, the genetically modified cell comprises a recombinant nucleic acid including a sequence that encodes (II) two hybrid proteins having a structure of A-C-B and A-C-D, respectively. In accordance with the present invention, A is a transmembrane domain that is not cleavable by the BoNT protease, B is a first fluorescent protein, C is a BoNT protease recognition and cleavage sequence, and D is a second fluorescent protein. Most preferably, A

comprises a transmembrane domain from synaptobrevin, synaptophysin, synapsin I, synapsin II, and/or synapsin III, and/or C comprises at least two of a BoNT/B, a BoNT/G, a BoNT/D, and a BoNT/F protease recognition and cleavage sequence. Where desired, at least one additional sequence may be provided that encodes a peptide linker that is disposed between at least one of A and C, A and B, C and B, and C and D, wherein the linker has a length of equal or less than 12 amino acids.

[0011] According to one aspect of the invention, the recombinant nucleic acid encodes the two hybrid proteins.

[0012] In exemplary nucleic acids, the hybrid protein having the structure of A-B-C-D is encoded by a sequence according to SEQ ID NO:1, the hybrid protein having the structure of A-C-B is encoded by a sequence according to SEQ ID NO:3, and the hybrid protein having the structure of A-C-B is encoded by a sequence according to SEQ ID NO:5.

[0013] According to one aspect of the present invention the cell comprises a nucleic acid that encodes the two hybrid proteins having the structure of A-C-B and A-C-D.

[0014] Various objects, features, aspects and advantages of the present invention will become more apparent from the following detailed description of preferred embodiments of the invention.

Brief Description of the Drawing

[0015] Prior Art Figures 1A-1B are known FRET assays for BoNT protease activity in which two fluorescent proteins are separated by a SNAP25 recognition and cleavage sequence.

[0016] Figures 2B-2B are schematic illustrations for intramolecular (2A) and intermolecular (2B) FRET assays for BoNT protease activity using the genetically modified cell according to the inventive subject matter.

[0017] Figures 3A-3B are exemplary vector maps for recombinant intramolecular (3A) and intermolecular (3B) FRET constructs of the genetically modified cell according to the inventive subject matter.

[0018] Figure 4A depicts exemplary FRET results for intramolecular (left panel) and intermolecular (right panel) FRET analysis of the genetically modified cell according to the inventive subject matter.

[0019] Figure 4B is a graphic representation of the results from the experiments of Figure 4A.

Detailed Description

[0020] According to the present invention a Genetically modified cell according to claims 1 or 8 and a kit according to claim 15 is provided.

[0021] It should be appreciated that the hybrid protein(s) that are formed in the so transfected cells include a transmembrane domain. Therefore, these proteins are expected to locate to intracellular vesicles and to so present a vesicle-bound substrate. Upon exposure of the cells with BoNT, heavy chain-mediated endocytosis of the BoNT into the transfected cell is followed by presentation of the light chain on the outer surface of the vesicle, allowing the protease activity of the light chain to cleave the cleavage sequence of the hybrid protein(s), thus reducing FRET and providing a quantifiable signal. Therefore, it should be appreciated that the cells and kit presented herein allow for a cell-based assay in which uptake, processing, and proteolytic activity can be monitored under conditions that closely resemble the natural conditions.

[0022] In contrast, as schematically depicted in **Prior Art Figure 1A,** a BoNT/A test system with a hybrid protein is shown in A. The hybrid protein has first and second fluorescence proteins (CFP and YFP, respectively) covalently coupled to the respective termini of an intermediate peptide sequence that also includes a SNAP-25 sequence (which is the substrate for the BoNT/A light chain protease). Excitation of the CFP results in FRET-mediated fluorescence emission of YFP, thus creating a specific spectral fluorescence signature as schematically illustrated in B. Upon incubation with BoNT/A, the SNAP-25 sequence is hydrolyzed and YFP is released from the hybrid molecule (the remainder of which is still bound to a membrane or other solid phase) as depicted in C. Alternatively, or additionally, excitation and emission may be followed only using YFP, which when separated from the hybrid protein, will ultimately be processed in the proteasome complex. Similarly, as shown in **Prior Art Figure 1B,** a hybrid protein has first and second fluorescence proteins (CFP and YFP, respectively) covalently coupled to the respective termini of an intermediate peptide sequence that also includes a SNAP-25 sequence. The hybrid protein is associated to the outside of the vesicle via the cysteine rich domain of the SNAP-25 sequence. Once more, upon cleavage of the SNAP-25 linker between the CFP and YFP, FRET is no longer available and fluorescence can be measured either as loss in FRET or ultimately loss in YFP as described above.

[0023] While such systems provide various advantages, it should be readily apparent that that where the SNAP-25 sequence is replaced by a synaptobrevin (VAMP), the presence of the transmembrane sub-domain in synaptobrevin will lead to physical separation of the CFP and YFP by the vesicle (or other) membrane, thus abolishing any FRET between the CFP and YFP as is shown in Figure 9B of U.S. Pat. App. No. 2006/0134722.

[0024] To overcome these difficulties, the inventors now have prepared hybrid molecules suitable for intramolecular FRET in which one fluorescent protein (or other reporter) is positioned between the transmembrane sub-domain and

the BoNT protease recognition and cleavage sequence, and wherein another fluorescent protein (or other reporter) is positioned following the BoNT protease recognition and cleavage sequence. Additionally, the inventors have also prepared pairs of hybrid molecules suitable for intermolecular FRET in which both hybrid molecules have a respective fluorescent protein coupled to respective sequences that include a transmembrane domain and a BoNT protease recognition and cleavage sequence.

[0025] As used herein, the term "transmembrane domain" refers to any molecular moiety that is capable of insertion into a plasma membrane in a manner such that at least a portion of the moiety extends into (and more typically across) the lipid bilayer. Thus, a moiety that only externally contacts (e.g., via ionic or electrostatic interaction) the outer surface of the plasma membrane is not considered a transmembrane domain under the definition provided herein. Thus, especially preferred transmembrane domains include hydrophobic polypeptide domains that extend into (and more typically across) the plasma membrane. Most typically, preferred transmembrane domains comprise a (typically recombinant) polypeptide. However, it should be recognized that various alternative elements (e.g., N-terminal palmitoylation) will also fall within the scope of the definition provided herein.

[0026] As also used herein, the term "BoNT recognition and cleavage sequence" refers to any molecular moiety that can be bound and cleaved by a BoNT protease. It is generally preferred that the BoNT recognition and cleavage sequence comprises a synaptobrevin polypeptide or portion thereof, which is typically a recombinant polypeptide.

[0027] According to a first aspect of the inventive subject matter, contemplated recombinant nucleic acids include a sequence that encodes (I) a hybrid protein having a structure of A-B-C-D. According to a second aspect of the inventive subject matter(II) at least one of two hybrid proteins having a structure of A-C-B and having a structure of A-C-D, respectively, where A is a transmembrane domain, B is a first fluorescent protein, C is a BoNT recognition and cleavage sequence, and D is a second fluorescent protein. Most preferably, where the sequence encodes two hybrid proteins, expression of the two hybrid proteins is under the control of respective promoters (typically, but not necessarily, having the same strength and same regulatory control mechanism).

[0028] Most typically, the transmembrane domain is selected such as to allow insertion of the recombinant protein(s) into the membrane of synaptic vesicles. Therefore, it is generally preferred that the transmembrane domain is the transmembrane domain of synaptobrevin, synaptophysin, synapsin I, synapsin II, and/or synapsin III, or any portion thereof that still confers anchoring of the recombinant protein into the membrane. However, in alternative aspects of the inventive subject matter, it is contemplated that various other transmembrane domains are also deemed suitable so long as such domains will anchor the recombinant protein to one or more intracellular membranes. There are numerous transmembrane domains known in the art, and all of those are deemed suitable for use herein. The person of ordinary skill in the art will readily be able to identify a domain as a transmembrane domain (e.g., via publication and description of the domain, or via computational domain analysis). Of course, suitable domains naturally occurring domains as well as mutated forms thereof (*e.g.,* forms with one or more transitions, transversions, insertions, deletions, inversions, etc.). Moreover, additionally contemplated transmembrane domain may also be entirely synthetic and based on computational analysis.

[0029] Similarly, it should be appreciated that the transmembrane domain may also be replaced by another polypeptide moiety that allows at least temporary anchoring of the hybrid protein to a membrane such that the remainder of the hybrid protein is exposed to the cytosol. Anchoring may be mediated by various (typically non-covalent) interactions, including ionic, hydrophobic, and/or electrostatic interactions. Still further contemplated transmembrane domains also include non-protein transmembrane domains. For example, especially preferred alternative transmembrane domains will include those in which a hydrophobic group (e.g., sterol, hydrocarbon, etc.) is attached to the protein, and particularly a palmitoyl group. Such groups may be added intracellularly (*e.g.,* via palmitoylation signal) or *in vitro* using various synthetic schemes.

[0030] It should further be appreciated that suitable transmembrane domains will preferably not include a BoNT protease cleavage site and/or a BoNT protease recognition site and thus only be acting as a transmembrane anchor for the recombinant protein. Therefore, suitable transmembrane domains may include full-length (or substantially full-length) synaptobrevin that has been sufficiently mutated to eliminate the cleavage site and/or recognition site. Alternatively, the synaptobrevin (or other transmembrane domain) may be truncated such that at least the cleavage site and/or recognition site is removed. Moreover, while the above is directed to single transmembrane domains, it should be appreciated that more than one transmembrane domains are also deemed appropriate (which are preferably coupled to each other via a spacer).

[0031] With respect to first and second fluorescent proteins it is generally contemplated that all known fluorescent proteins are suitable for use herein so long as such proteins can be used as specific and distinct signal generation moieties. However, it is particularly preferred that the signal generation moieties are fluorescent proteins that are suitable for FRET. For example, first and second fluorescent proteins can be Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) and their respective modifications, respectively. Of course, and as already noted above, the fluorescent proteins may be modified to include one or more specific characteristics (*e.g.*, spectral) or be truncated to a specific size. Among other choices, contemplated fluorescent proteins include various blue fluorescent proteins (*e.g.*,

EBFP, EBFP2, Azurite, mKalama1), various cyan fluorescent proteins (*e.g.*, ECFP, Cerulean, CyPet), various green fluorescent proteins (*e.g.*, AcGFP1, ZsGreen1), and various yellow fluorescent protein derivatives (*e.g.*, YFP, Citrine, Venus, YPet).

[0032] Similarly, it should be appreciated that the BoNT protease recognition and cleavage sequence may vary considerably, so long as such sequence is still recognized and hydrolyzed by a BoNT light chain. For example, the BoNT protease recognition and cleavage sequence may be of human, rat, or murine origin, may be present in oligo-multimeric form, and may be further specifically modified to facilitate or at least partially inhibit cleavage. Alternatively, the BoNT protease recognition and cleavage sequence may also be modified to allow identification of one or more specific BoNT subtypes (e.g., BoNT/B, D, F, and/or G, as well tetanus toxin) by preferential or exclusive cleavage. Of course, it should be recognized that all isoforms and mutants of BoNT protease recognition and cleavage sequences are also deemed suitable for use in conjunction with the teachings presented herein so long as such forms and mutants are also cleavable by one or more BoNT proteases. For example, suitable protease recognition and cleavage sequences include those from VAMP (Synaptobrevin) 1, 2, 3, 4, 5, 6, 7, or 8, and exemplary sequences are listed below where the recognition and cleavage domain is in regular type font, the transmembrane domain is in cursive type font, and where the actual cleavage positions for the respective BoNT proteases are underlined (QK: BoNT/F; KL: BoNT/D; QF: BoNT/B and TeTN; AA: BoNT/G):

[0033] Rat Vamp2 Protein sequence (SEQ ID NO:7):

MSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY*WWKNLKMMIILGVICAIILIIIVYFST* SEQ ID NO:7

[0034] Mouse Vamp2 Protein sequence (SEQ ID NO:8):

MSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY*WWKNLKMMIILGVICAIILIIIVYFST* (SEQ ID NO:8)

[0035] Human Vamp2 Protein sequence (SEQ ID NO:9):

MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY*WWKNLKMMIILGVICAIILIIIVYFST* (SEQ ID NO:9)

[0036] Of course, it should be noted that the above sequences merely serve as examples for the sequences from which the transmembrane domain and the BoNT protease recognition and cleavage sequences can be obtained from. Thus, it is also noted that numerous alternative sequences other than synaptobrevin are also contemplated particularly if they can be cleaved by a naturally occurring or a synthetic or designer BoNT, including SNAP-25 and mutant forms thereof.

[0037] It should further be appreciated that one or more of the transmembrane domain, the first and second fluorescent proteins, and the BoNT protease recognition and cleavage domain may be truncated while retaining the respective function (*i.e.,* transmembrane anchor, fluorescence, BoNT protease recognition and cleavage). Moreover, it should be appreciated that one or more amino acids in the above elements may be deleted or replaced by one or more other amino acids, typically in a conserved fashion.

[0038] Moreover, it is especially contemplated that the additional amino acids may be added as spacers between one or more of the transmembrane domain, the first and second fluorescent proteins, and the BoNT protease recognition and cleavage domain. Such spacers may be included to provide further steric flexibility, increase distance between the elements, etc. Typically, suitable spacers will have a length of between 1-100 amino acids, more typically between 2-50 amino acids, and most typically between 3-12 amino acids. Where the recombinant protein is used for FRET assays, shorter spacers are generally preferred. Still further, it is noted that additional domains may be provided to impart further desired functions. For example, suitable additional domains will include affinity tags for ease of isolation or antibody-based labeling, cell trafficking to direct the recombinant protein into a desired compartment, etc.

[0039] With respect to the transfected cells expressing the hybrid protein it is generally preferred that the cell is stably transfected. Nevertheless, transient transfection is also contemplated. There are numerous promoter structures known in the art, and all of those are generally deemed suitable for use herein. However, it is especially preferred that the expression is inducible from the promoter. In further contemplated aspects, expression may also be constitutively. **Figure 3A** depicts an exemplary vector map for an expression construct of a hybrid protein having a structure of A-B-C-D, and **Figure 3B** depicts an exemplary vector map for expression of two hybrid proteins having a structure of A-C-B and A-C-D, respectively.

[0040] Particularly preferred cells for transfection include neuronal cells (*e.g.*, astrocytes, dendrocytes, glia cells, etc.) and stem cells (*e.g.,* adult pluripotent, or adult germ line layer, or adult progenitor). However, numerous other non-neuronal cells, including human, rodent, insect cells, and even yeast and bacterial cells are also contemplated herein.

[0041] Consequently, the inventors contemplate genetically modified cells for a cell-based method of measuring protease activity of a BoNT protease in which in one step a transfected cell is provided that produces (I) a hybrid protein having a structure of A-B-C-D or (II) two hybrid proteins having a structure of A-C-B and A-C-D, respectively, wherein A is a transmembrane domain, B is a first fluorescent protein, C is a BoNT recognition and cleavage sequence, and D is a second fluorescent protein. In exemplary aspects of the inventive subject matter, the hybrid protein having a structure of A-B-C-D has a sequence according to SEQ ID NO:2, which is preferably encoded by a nucleic acid having sequence according to SEQ ID NO:1. Where the hybrid proteins have a structure of A-C-B and A-C-D, the protein sequences will preferably be as shown in SEQ ID NO:4 and SEQ ID NO:6, which are preferably encoded by a nucleic acid having sequence according to SEQ ID NO:3 and SEQ ID NO:5, respectively. Of course, and as already noted earlier, all mutant forms of the above sequences are also expressly contemplated herein, so long as such mutant forms retain the respective functions as noted above. In another step, the transfected cell is contacted with a BoNT protease under conditions to allow the cell to take up the BoNT protease, and in yet another step, fluorescence is measured from at least one of the first and second fluorescent proteins in the transfected cell.

[0042] Depending on the particular requirements and conditions, cell based assays using the genetically modified cells of the present invention may be performed as depicted in **Figure 2A** in which the hybrid protein is a single polypeptide chain having an N-terminal transmembrane domain, followed by a CFP, which is in turn followed by a BoNT protease recognition and cleavage sequence, which is in turn followed by a (preferably terminal) YFP. Expression of the hybrid protein and subsequent translocation of the hybrid protein to the membrane of an intracellular vesicle will result in the presentation of the hybrid protein on the outside of the vesicle. The protease activity of BoNT/B will then intracellularly cleave the cleavage sequence, thus releasing the YFP from the hybrid protein. Consequently, quenching is reduced and fluorescence of the YFP is detectable in diffused form from the cell.

[0043] Alternatively, as shown in **Figure 2B,** two separate hybrid proteins are formed in the cell, each having an N-terminal transmembrane domain, followed by a BoNT protease recognition and cleavage sequence, which is in turn followed by a (preferably terminal) YFP and CFP, respectively. Expression of the hybrid proteins and subsequent translocation of the hybrid proteins to the membrane of an intracellular vesicle will result in the presentation of the hybrid proteins on the outside of the vesicle. The protease activity of BoNT/B will then intracellularly cleave the cleavage sequences, thus releasing YFP and CFP from the hybrid protein. Consequently, quenching is reduced and fluorescence of the YFP and CFP is detectable in diffused form from the cell. Remarkably, the respective hybrid proteins co-locate on the vesicular membrane in such a manner as to allow for FRET. Exemplary results for such assays are depicted in the calculated fluorescence microphotographs of **Figure 4A** and the corresponding bar graph representations of **Figure 4B.** As can be readily taken from these figures, the FRET assay performed well in the intermolecular FRET assay and less satisfactorily in the intramolecular FRET assay. However, it is expected that routine experimentation will also provide satisfactory intramolecular FRET assay results.

Examples

Cloning of Intramolecular Construct

[0044] The intramolecular FRET construct, pMD0031 (Figure 3A), was constructed in pEGFP-C1 (Clontech, Mountain View, CA). Three DNA fragments- an N-terminal fragment of rat Vamp2 from the start to amino acid 92, full length YFP without a stop codon, and a C-terminal fragment of rat Vamp2 from amino acid 93 to the stop- were amplified by polymerase chain reaction (PCR). An EcoRI restriction site was engineered onto the 5' end of the N-terminal Vamp2 fragment and a SerGlyGly (TCGGGAGGC) linker and the first 12 nucleotides of the YFP were engineered onto the 3' end. The YFP fragment had the last 13 nucleotides of the N-terminal Vamp2 fragment and the same SerGlyGly linker as the N-terminal Vamp2 fragment engineered onto the 5' end and a second SerGlyGly (AGCGGCGGT) linker and the first 9 nucleotides of the C-terminal Vamp2 fragment engineered onto the 3' end. The C-terminal Vamp2 fragment had the last 12 nucleotides of YFP without a stop and the same SerGlyGly linker as the YFP fragment engineered onto the 5' end and a BamHI restriction site engineered onto the 3' end.

[0045] These three fragments were then combined using splice overlap extension (SOE) PCR to create a single fragment consisting of an EcoRI restriction site, the N-terminal fragment of rat Vamp2 (amino acids 1-92), a SerGlyGly linker, YFP without a stop, a second SerGlyGly linker, the C-terminal fragment of rat Vamp2 (amino acids 93-stop), and an BamHI restriction site. This fragment and pECFP-C1 were then digested with EcoRI and BamHI, ligated together, and transformed into DH5α E. coli. The final construct insert was then fully sequenced.

Cloning of Intermolecular Construct

[0046] The intermolecular FRET construct, pMD0034 (Figure 3B), was constructed in pBudCE4.1 (Invitrogen, Carlsbad, CA). The YFP rat Vamp2 fusion was generated by amplifying two fragments by PCR. The first fragment was YFP without a stop with an engineered HindIII restriction site on the 5' end and a SerGlyGly linker (AGTGGAGGC) and the first 9 nucleotides of rat Vamp2 engineered on the 3' end. The second fragment was full length rat Vamp2 with the last 9 nucleotides of YFP and the same SerGlyGly linker engineered onto the 5' end and an XbaI restriction site engineered onto the 3' end. These two fragments were then combined using SOE PCR to create a YFP, SerGlyGly linker, full length Vamp2 fragment. The fragment and pBudCE4.1 was then digested with HindIII and XbaI, ligated together, and transformed into DH5 α E. coli. The CFP rat Vamp2 fusion was created similarly but contained a CFP without a stop, a NotI restriction site on the 5' end, and a KpnI site on the 3' end. The final construct was then fully sequenced.

Cell Culture and FRET Assay

[0047] Analysis of FRET efficiency, YFP/CFP fluorescence ratios, and BoNT/B sensitivities of the BoNT/B reporters was performed in cells in vitro. More specifically, Neuro2A cells were grown in a 96-well plate to 70% confluency (~2000 cells/well) and transiently transfected using Lipofectamine 2000 (Invitrogen), with the intra- or intermolecular BoNT/B reporters. After 24 h, cells were incubated in the presence or absence of 25 nM BoNT/B at 37 °C for 72 h in 100 μl of phenol red-free MEM medium.

[0048] Semi-automated FRET or total YFP and CFP fluorescence measurements were performed using a Nikon TE2000-U fluorescent microscope with 200x magnification and Nikon NIS Elements 3.4 software. For FRET measurements, coefficients -A and -B (acceptor and donor) were calculated at 0.03 and 0.73 respectively, using a specific bleed-through method. Figure 4A depicts randomly selected fields pseudo-colored for FRET efficiency or the YFP/CFP fluorescence ratio. YFP/CFP ratios were calculated from emissions collected upon direct excitement of each fluorophore. As can be seen from the graphic representation in Figure 4B, the intermolecular BoNT/B reporter approach was significantly more sensitive for detection of BoNT/B in living cells. 30 randomly selected cells per condition were analyzed for FRET efficiency (Figure 4A, left panels) or YFP/CFP fluorescence ratios (Figure 4A, right panels) in the presence or absence of 25 nM BoNT/B. Indeed, such results were entirely unexpected as effective intermolecular FRET not only required balanced expression of the two fluorescent proteins, but also co-location of the recombinant proteins in corresponding quantities. The average signal from the 30 cells from 5 microscopic fields on 3 different wells is shown. Cells exhibiting over-saturated fluorescence were excluded.

SEQUENCE LISTING

[0049]

<110> BioMadison, Inc.

<120> Resonance Energy Transfer Assay with Synaptobrevin Substrate Moiety

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ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc 180

ctcgtgacca ccctgacctg gggcgtgcag tgcttcagcc gctaccccga ccacatgaag 240

cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc 300

ttcaaggacg acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg 360

gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac 420

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gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac 600

tacctgagca cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc 660

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<223> C-terminal portion of VAMP2 including transmembrane domain and no cleavage sites

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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
            20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60
```

```
Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
225                 230                 235                 240

Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Met Ser Ala Thr Ala
                245                 250                 255

Ala Thr Val Pro Pro Ala Ala Pro Ala Gly Glu Gly Gly Pro Pro Ala
            260                 265                 270

Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu Gln Gln Thr Gln Ala
        275                 280                 285

Gln Val Asp Glu Val Val Asp Ile Met Arg Val Asn Val Asp Lys Val
    290                 295                 300

Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala
305                 310                 315                 320
```

```
Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys
                325                 330                 335

Arg Lys Tyr Ser Gly Gly Met Val Ser Lys Gly Glu Glu Leu Phe Thr
            340                 345                 350

Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His
        355                 360                 365

Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys
    370                 375                 380

Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp
385                 390                 395                 400

Pro Thr Leu Val Thr Thr Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg
                405                 410                 415

Tyr Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro
            420                 425                 430

Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn
        435                 440                 445

Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn
    450                 455                 460

Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu
465                 470                 475                 480

Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met
                485                 490                 495

Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His
            500                 505                 510

Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn
        515                 520                 525

Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu
    530                 535                 540

Ser Tyr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His
545                 550                 555                 560

Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met
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565 570 575

Asp Glu Leu Tyr Lys Ser Gly Gly Trp Trp Lys Asn Leu Lys Met Met
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Tyr Phe Ser Thr
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<220>
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gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac 420

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gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac 600

tacctgagct accagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc 660

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ggaggcatgt cggctaccgc tgccaccgtc ccgcctgccg ccccggccgg cgagggtggc 780

ccccctgcac ctcctccaaa tcttaccagt aacaggagac tgcagcagac ccaggcccag 840

gtggatgagg tggtggacat catgagggtg aatgtggaca aggtcctgga gcgagaccag 900

aagctatcgg aactggatga tcgcgcagat gccctccagg caggggcctc ccagtttgaa 960

acaagtgcag ccaagctcaa gcgcaaatac tggtggaaaa acctcaagat gatgatcatc 1020

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            20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60

Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95
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Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
225                 230                 235                 240

Gly Gly Met Ser Ala Thr Ala Ala Thr Val Pro Pro Ala Ala Pro Ala
                245                 250                 255

Gly Glu Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg
            260                 265                 270

Arg Leu Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met
        275                 280                 285

Arg Val Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu
    290                 295                 300

Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu
305                 310                 315                 320

Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys
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Met Met Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile
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<223> Full-length VAMP2 with transmembrane domain and cleavage sites

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ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc 180

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cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc 300

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aagctggagt acaactacat cagccacaac gtctatatca ccgccgacaa gcagaagaac 480

ggcatcaagg ccaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc 540

gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac 600

tacctgagca cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc 660

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aagctatcgg aactggatga tcgcgcagat gccctccagg caggggcctc ccagtttgaa 960

acaagtgcag ccaagctcaa gcgcaaatac tggtggaaaa acctcaagat gatgatcatc 1020

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<210> 6
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<212> PRT
<213> Artificial

<220>
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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
            20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60

Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125
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Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
225                 230                 235                 240

Gly Gly Met Ser Ala Thr Ala Ala Thr Val Pro Pro Ala Ala Pro Ala
                245                 250                 255

Gly Glu Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg
            260                 265                 270

Arg Leu Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met
        275                 280                 285

Arg Val Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu
    290                 295                 300

Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu
305                 310                 315                 320

Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys
                325                 330                 335

Met Met Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile
            340                 345                 350

Ile Val Tyr Phe Ser Thr
        355
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<212> PRT
<213> Rattus norvegicus

<400> 7

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Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu
            20                  25                  30

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
        35                  40                  45

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
    50                  55                  60

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
65                  70                  75                  80

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
                85                  90                  95

Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
            100                 105                 110

Tyr Phe Ser Thr
        115
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<210> 8
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<213> Mus musculus

<400> 8

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Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu
            20                  25                  30

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
        35                  40                  45

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
    50                  55                  60

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
65                  70                  75                  80

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
                85                  90                  95

Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
            100                 105                 110

Tyr Phe Ser Thr
        115
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<210> 9
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<212> PRT
<213> Homo sapiens

<400> 9

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1               5                  10                  15

Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu
            20                  25                  30

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
        35                  40                  45

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
    50                  55                  60

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
65                  70                  75                  80

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
                85                  90                  95

Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
            100                 105                 110

Tyr Phe Ser Thr
        115
```

Claims

1. A genetically modified cell, comprising:

   a nucleic acid comprising a nucleotide sequence that encodes a hybrid protein having a structure of A-B-C-D, wherein A comprises a transmembrane domain that is not cleavable by a BoNT protease, B comprises a first fluorescent protein, C comprises a BoNT protease recognition and cleavage sequence, and D comprises a second fluorescent protein; wherein the transmembrane domain inserts across a lipid membrane of the cell such that B, C, and D are located on the same side of the lipid membrane,
   and
   wherein the nucleic acid comprises a promoter element suitable for expression of the hybrid protein in the genetically modified cell.

2. The genetically modified cell of claim 1, wherein the cell is stably transfected with the nucleic acid comprising the nucleotide sequence.

3. The genetically modified cell of claim 1, wherein the cell is selected from the group consisting of a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, and a stem cell.

4. The genetically modified cell of claim 1, wherein the transmembrane domain is selected from one or more of the group consisting of synaptobrevin, synaptophysin, synapsin I, synapsin II, and synapsin III.

5. The genetically modified cell of claim 1, wherein the BoNT protease recognition and cleavage sequence is selected from at least one of the group consisting of a BoNT/B protease recognition and cleavage sequence, a BoNT/G protease recognition and cleavage sequence, a BoNT/D protease recognition and cleavage sequence, and a BoNT/F protease recognition and cleavage sequence.

6. The genetically modified cell of claim 1, wherein the first fluorescent protein is selected from the group consisting of blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, and yellow fluorescent protein.

7. The genetically modified cell of claim 1, wherein the second fluorescent protein is selected from the group consisting of blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, and yellow fluorescent protein.

8. A genetically modified cell, comprising:

a nucleic acid that comprises a nucleotide sequence encoding a first hybrid protein and a second hybrid protein, wherein the first hybrid protein has a structure of A-C-B and the second hybrid protein has a structure of A-C-D, wherein A comprises a transmembrane domain that is not cleavable by the BoNT protease, B comprises a first fluorescent protein, C comprises a BoNT protease recognition and cleavage sequence, and D comprises a second fluorescent protein; wherein the transmembrane domain inserts across a lipid membrane of the cell such that B, C, and D are located on the same side of the lipid membrane,
and
wherein the nucleic acid comprises a first promoter element suitable for expression of the first hybrid protein and a second promoter element suitable for expression of the second hybrid protein in the genetically modified cell.

9. The genetically modified cell of claim 8, wherein the cell is stably transfected with the nucleic acid.

10. The genetically modified cell of claim 8, wherein the cell is selected from the group consisting of a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, and a stem cell.

11. The genetically modified cell of claim 8, wherein the transmembrane domain is selected from one or more of the group consisting of synaptobrevin, synaptophysin, synapsin I, synapsin II, and synapsin III.

12. The genetically modified cell of claim 8, wherein the BoNT protease recognition and cleavage sequence is selected from at least one of the group consisting of a BoNT/B protease recognition and cleavage sequence, a BoNT/G protease recognition and cleavage sequence, a BoNT/D protease recognition and cleavage sequence, and a BoNT/F protease recognition and cleavage sequence.

13. The genetically modified cell of claim 8, wherein the first fluorescent protein is selected from the group consisting of blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, and yellow fluorescent protein.

14. The genetically modified cell of claim 8, wherein the second fluorescent protein is selected from the group consisting of blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, and yellow fluorescent protein.

15. A kit for a cell-based assay for botulinum toxin characterization comprising:
the genetically modified cell according to anyone of claims 1 - 14 ; and instructions for use of the transfected cell in an assay for a botulinum neurotoxin.

Patentansprüche

1. Genetisch veränderte Zelle, die umfasst:

eine Nukleinsäure, die eine Nukleotidsequenz umfasst, die ein Hybridprotein mit einer Struktur A-B-C-D codiert, wobei A eine Transmembrandomäne umfasst, die durch eine BoNT-Protease nicht spaltbar ist, B ein erstes Fluoreszenzprotein umfasst, C eine BoNT-Proteaseerkennungs- und -spaltungssequenz umfasst und D ein zweites Fluoreszenzprotein umfasst, wobei die Transmembrandomäne in der Weise über eine Lipidmembran der Zelle eingefügt ist, dass sich B, C und D auf derselben Seite der Lipidmembran befinden, und
wobei die Nukleinsäure ein Promotorelement, das für die Expression des Hybridproteins in der genetisch veränderten Zelle geeignet ist, umfasst.

2. Genetisch veränderte Zelle gemäß Anspruch 1, wobei die Zelle mit einer Nukleinsäure, die die Nukleotidsequenz umfasst, stabil transfiziert ist.

3. Genetisch veränderte Zelle gemäß Anspruch 1, wobei die Zelle aus der Gruppe ausgewählt ist, die aus einer Nervenzelle, aus einer neuroendokrinen Tumorzelle, aus einer Hybridzelle und aus einer Stammzelle besteht.

4. Genetisch veränderte Zelle gemäß Anspruch 1, wobei die Transmembrandomäne aus einem oder aus mehreren der Gruppe ausgewählt ist, die aus Synaptobrevin, Synaptophysin, Synapsin I, Synapsin II und Synapsin III besteht.

5. Genetisch veränderte Zelle gemäß Anspruch 1, wobei die BoNT-Proteaseerkennungs- und -spaltungssequenz wenigstens aus einem der Gruppe ausgewählt ist, die aus einer BoNT/B-Proteaseerkennungs- und -spaltungssequenz, aus einer BoNT/G-Proteaseerkennungs- und -spaltungssequenz, aus einer BoNT/D-Proteaseerkennungs- und -spaltungssequenz und aus einer BoNT/F-Proteaseerkennungs- und -spaltungssequenz besteht.

6. Genetisch veränderte Zelle gemäß Anspruch 1, wobei das erste Fluoreszenzprotein aus der Gruppe ausgewählt ist, die aus blauem Fluoreszenzprotein, aus Zyan-Fluoreszenzprotein, aus grünem Fluoreszenzprotein und aus gelbem Fluoreszenzprotein besteht.

7. Genetisch veränderte Zelle gemäß Anspruch 1, wobei das zweite Fluoreszenzprotein aus der Gruppe ausgewählt ist, die aus blauem Fluoreszenzprotein, aus Zyan-Fluoreszenzprotein, aus grünem Fluoreszenzprotein und aus gelbem Fluoreszenzprotein besteht.

8. Genetisch veränderte Zelle, die umfasst:

eine Nukleinsäure, die eine Nukleotidsequenz umfasst, die ein erstes Hybridprotein und ein zweites Hybridprotein codiert,
wobei das erste Hybridprotein eine Struktur A-C-B aufweist und wobei das zweite Hybridprotein eine Struktur A-C-D aufweist, wobei A eine Transmembrandomäne umfasst, die durch die BoNT-Protease nicht spaltbar ist, B ein erstes Fluoreszenzprotein umfasst, C eine BoNT-Proteaseerkennungs- und -spaltungssequenz umfasst und D ein zweites Fluoreszenzprotein umfasst; wobei die Transmembrandomäne in der Weise über eine Lipidmembran der Zelle eingefügt ist, dass sich B, C, und D auf derselben Seite der Lipidmembran befinden, und
wobei die Nukleinsäure ein erstes Promotorelement, das für die Expression des ersten Hybridproteins geeignet ist, und ein zweites Promotorelement, das für die Expression des zweiten Hybridproteins in der genetisch veränderten Zelle geeignet ist, umfasst.

9. Genetisch veränderte Zelle gemäß Anspruch 8, wobei die Zelle mit der Nukleinsäure stabil transfiziert ist.

10. Genetisch veränderte Zelle gemäß Anspruch 8, wobei die Zelle aus der Gruppe ausgewählt ist, die aus einer Nervenzelle, aus einer neuroendokrinen Tumorzelle, aus einer Hybridzelle und aus einer Stammzelle besteht.

11. Genetisch veränderte Zelle gemäß Anspruch 8, wobei die Transmembrandomäne aus einem oder aus mehreren der Gruppe ausgewählt ist, die aus Synaptobrevin, Synaptophysin, Synapsin I, Synapsin II und Synapsin III besteht.

12. Genetisch veränderte Zelle gemäß Anspruch 8, wobei die BoNT-Proteaseerkennungs- und -spaltungssequenz wenigstens aus einem der Gruppe ausgewählt ist, die aus einer BoNT/B-Proteaseerkennungs- und -spaltungssequenz, aus einer BoNT/G-Proteaseerkennungs- und -spaltungssequenz, aus einer BoNT/D-Proteaseerkennungs- und -spaltungssequenz und aus einer BoNT/F-Proteaseerkennungs- und -spaltungssequenz besteht.

13. Genetisch veränderte Zelle gemäß Anspruch 8, wobei das erste Fluoreszenzprotein aus der Gruppe ausgewählt ist, die aus blauem Fluoreszenzprotein, aus Zyan-Fluoreszenzprotein, aus grünem Fluoreszenzprotein und aus gelbem Fluoreszenzprotein besteht.

14. Genetisch veränderte Zelle gemäß Anspruch 8, wobei das zweite Fluoreszenzprotein aus der Gruppe ausgewählt ist, die aus blauem Fluoreszenzprotein, aus Zyan-Fluoreszenzprotein, aus grünem Fluoreszenzprotein und aus gelbem Fluoreszenzprotein besteht.

15. Ausrüstung für einen zellenbasierten Biotest für die Botulinustoxin-Charakterisierung, wobei die Ausrüstung umfasst: die genetisch veränderte Zelle gemäß einem der Ansprüche 1-14; und Anweisungen zur Verwendung der transfizierten Zelle in einem Biotest für ein Botulinusneurotoxin.

Revendications

1. Cellule génétiquement modifiée comprenant :

   un acide nucléique comprenant une séquence nucléotidique qui code pour une protéine hybride ayant une structure A-B-C-D,
   où A comprend un domaine transmembranaire qui n'est pas clivable par une protéase de la BoNT, B comprend une première protéine fluorescente, C comprend une séquence de reconnaissance et de clivage par la protéase de la BoNT et D comprend une deuxième protéine fluorescente ; où le domaine transmembranaire s'insère au travers d'une membrane lipidique de la cellule d'une manière telle que B, C et D sont situés du même côté de la membrane lipidique,
   et
   où l'acide nucléique comprend un élément promoteur approprié pour l'expression de la protéine hybride dans la cellule génétiquement modifiée.

2. Cellule génétiquement modifiée de la revendication 1, la cellule étant transfectée de manière stable avec l'acide nucléique qui comprend la séquence nucléotidique.

3. Cellule génétiquement modifiée de la revendication 1, la cellule étant sélectionnée dans le groupe consistant en une cellule neuronale, une cellule tumorale neuroendocrine, une cellule hybride et une cellule souche.

4. Cellule génétiquement modifiée de la revendication 1, le domaine transmembranaire étant sélectionné parmi un ou plusieurs du groupe consistant en la synaptobrévine, la synaptophysine, la synapsine I, la synapsine II et la synapsine III.

5. Cellule génétiquement modifiée de la revendication 1, la séquence de reconnaissance et de clivage par la protéase de la BoNT étant sélectionnée parmi une au moins du groupe consistant en une séquence de reconnaissance et de clivage par la protéase de la BoNT/B, une séquence de reconnaissance et de clivage par la protéase de la BoNT/G, une séquence de reconnaissance et de clivage par la protéase de la BoNT/D et une séquence de reconnaissance et de clivage par la protéase de la BoNT/F.

6. Cellule génétiquement modifiée de la revendication 1, la première protéine fluorescente étant sélectionnée dans le groupe consistant en une protéine fluorescente bleue, une protéine fluorescente cyan, une protéine fluorescente verte et une protéine fluorescente jaune.

7. Cellule génétiquement modifiée de la revendication 1, la deuxième protéine fluorescente étant sélectionnée dans le groupe consistant en une protéine fluorescente bleue, une protéine fluorescente cyan, une protéine fluorescente verte et une protéine fluorescente jaune.

8. Cellule génétiquement modifiée comprenant :

   un acide nucléique comprenant une séquence nucléotidique qui code pour une première protéine hybride et une deuxième protéine hybride,
   la première protéine hybride ayant une structure A-C-B et la deuxième protéine hydride ayant une structure A-C-D, où A comprend un domaine transmembranaire qui n'est pas clivable par la protéase de la BoNT, B comprend une première protéine fluorescente, C comprend une séquence de reconnaissance et de clivage par la protéase de la BoNT et D comprend une deuxième protéine fluorescente ; où le domaine transmembranaire s'insère au travers d'une membrane lipidique de la cellule d'une manière telle que B, C et D sont situés du même côté de la membrane lipidique,
   et
   où l'acide nucléique comprend un premier élément promoteur approprié pour l'expression de la première protéine hybride et un deuxième élément promoteur approprié pour l'expression de la deuxième protéine hybride dans la cellule génétiquement modifiée.

9. Cellule génétiquement modifiée de la revendication 8, la cellule étant transfectée de manière stable avec l'acide nucléique.

10. Cellule génétiquement modifiée de la revendication 8, la cellule étant sélectionnée dans le groupe qui consiste en

une cellule neuronale, une cellule tumorale neuroendocrine, une cellule hybride et une cellule souche.

11. Cellule génétiquement modifiée de la revendication 8, le domaine transmembranaire étant sélectionné parmi un ou plusieurs du groupe consistant en la synaptobrévine, la synaptophysine, la synapsine I, la synapsine II et la synapsine III.

12. Cellule génétiquement modifiée de la revendication 8, la séquence de reconnaissance et de clivage par la protéase de la BoNT étant sélectionnée parmi une au moins du groupe consistant en une séquence de reconnaissance et de clivage par la protéase de la BoNT/B, une séquence de reconnaissance et de clivage par la protéase de la BoNT/G, une séquence de reconnaissance et de clivage par la protéase de la BoNT/D et une séquence de reconnaissance et de clivage par la protéase de la BoNT/F.

13. Cellule génétiquement modifiée de la revendication 8, la première protéine fluorescente étant sélectionnée dans le groupe consistant en une protéine fluorescente bleue, une protéine fluorescente cyan, une protéine fluorescente verte et une protéine fluorescente jaune.

14. Cellule génétiquement modifiée de la revendication 8, la deuxième protéine fluorescente étant sélectionnée dans le groupe consistant en une protéine fluorescente bleue, une protéine fluorescente cyan, une protéine fluorescente verte et une protéine fluorescente jaune.

15. Kit de test sur cellules pour la caractérisation d'une toxine botulinique, comprenant :
la cellule génétiquement modifiée selon l'une quelconque des revendications 1 - 14; et des instructions concernant l'utilisation de la cellule pour l'analyse d'une neurotoxine botulinique.

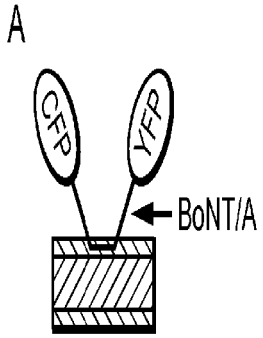
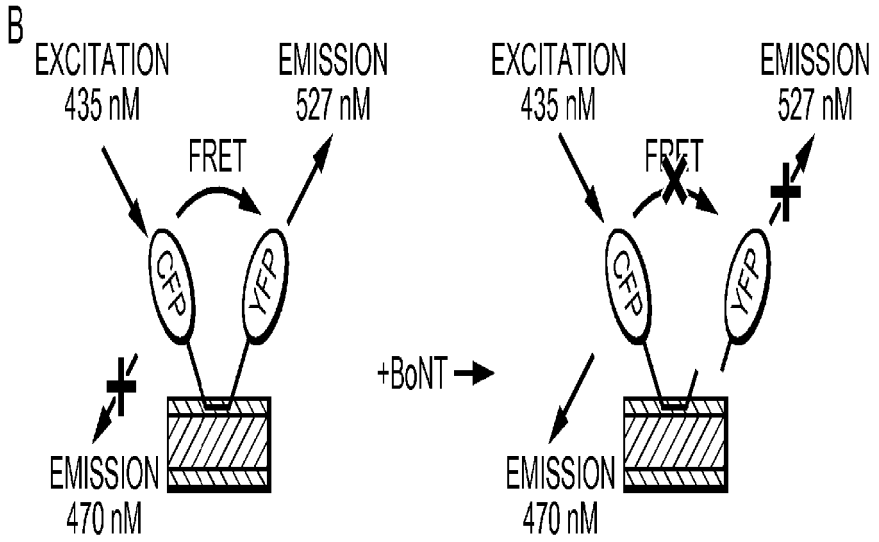


FIG. 1A
PRIOR ART



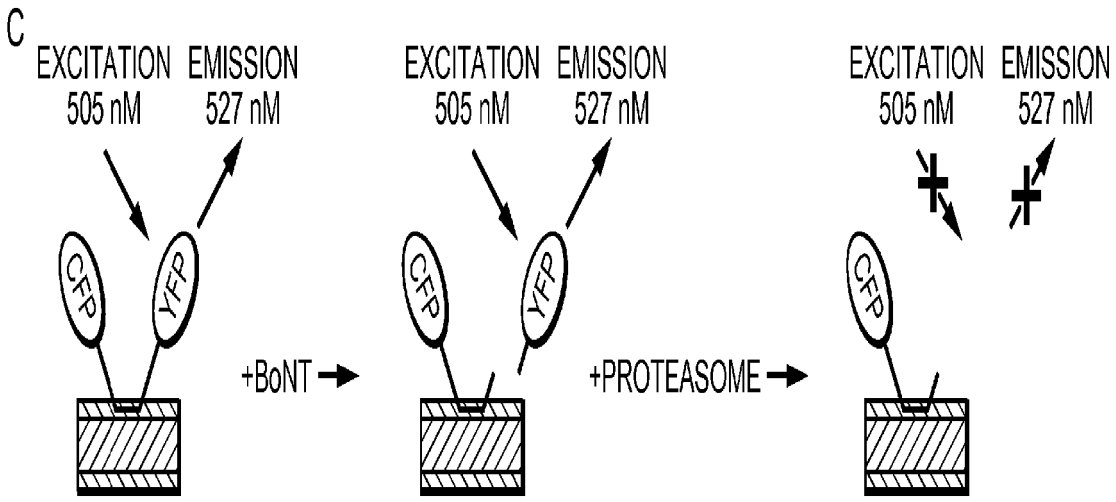
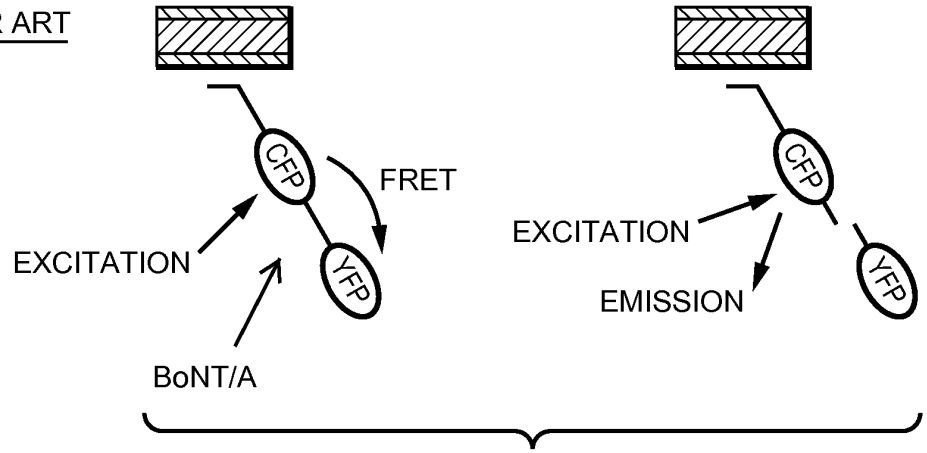


FIG. 1B
PRIOR ART



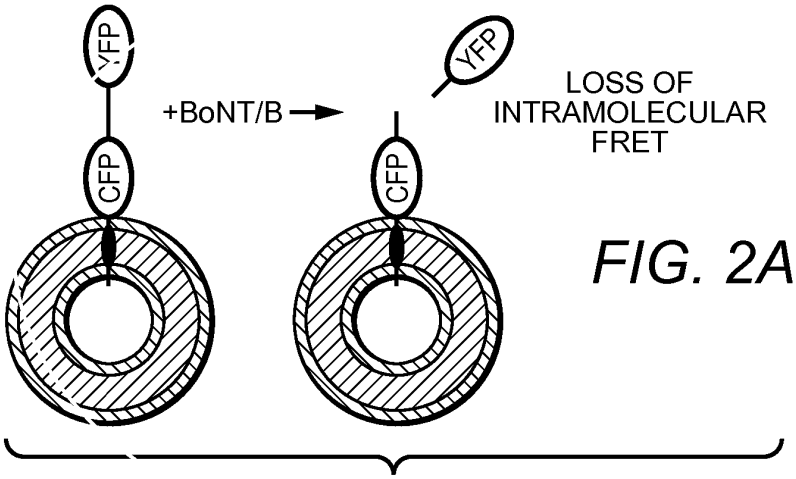


FIG. 2A

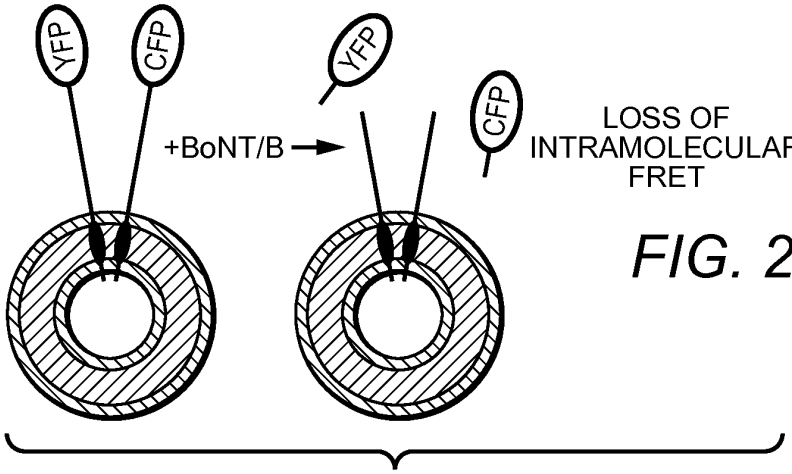


FIG. 2B

FIG. 3A

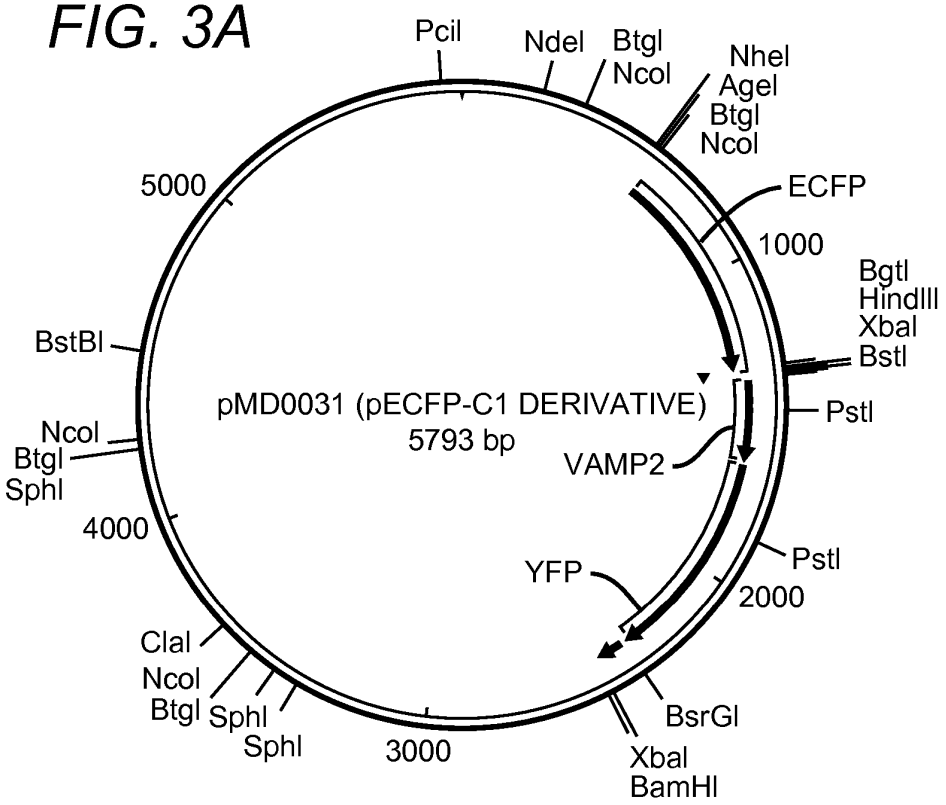

PciI
NdeI
BtgI
NcoI
NheI
AgeI
BtgI
NcoI
ECFP
1000
BgtI
HindIII
XbaI
BstI
PstI
pMD0031 (pECFP-C1 DERIVATIVE)
5793 bp
VAMP2
PstI
2000
YFP
BsrGI
XbaI
BamHI
3000
ClaI
NcoI
BtgI
SphI
SphI
4000
NcoI
BtgI
SphI
BstBI
5000

FIG. 3B

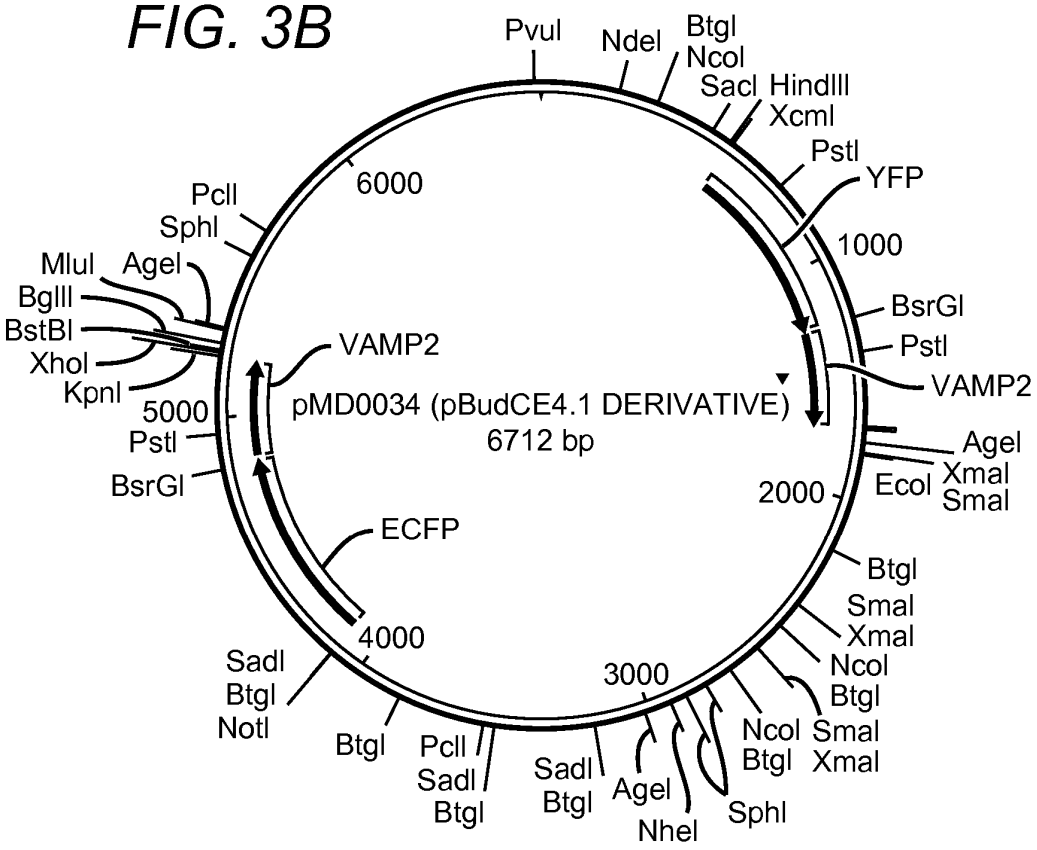

PvuI
NdeI
BtgI
NcoI
SacI
HindIII
XcmI
PstI
YFP
1000
BsrGI
PstI
VAMP2
AgeI
XmaI
SmaI
EcoI
2000
BtgI
SmaI
XmaI
NcoI
BtgI
NcoI
SmaI
XmaI
BtgI
3000
SadI
BtgI
NheI
AgeI
SadI
BtgI
PciI
SadI
BtgI
BtgI
4000
SadI
BtgI
NotI
ECFP
BsrGI
PstI
5000
pMD0034 (pBudCE4.1 DERIVATIVE)
6712 bp
VAMP2
XhoI
KpnI
BstBI
BglII
MluI
AgeI
PciI
SphI
6000

FIG. 4A

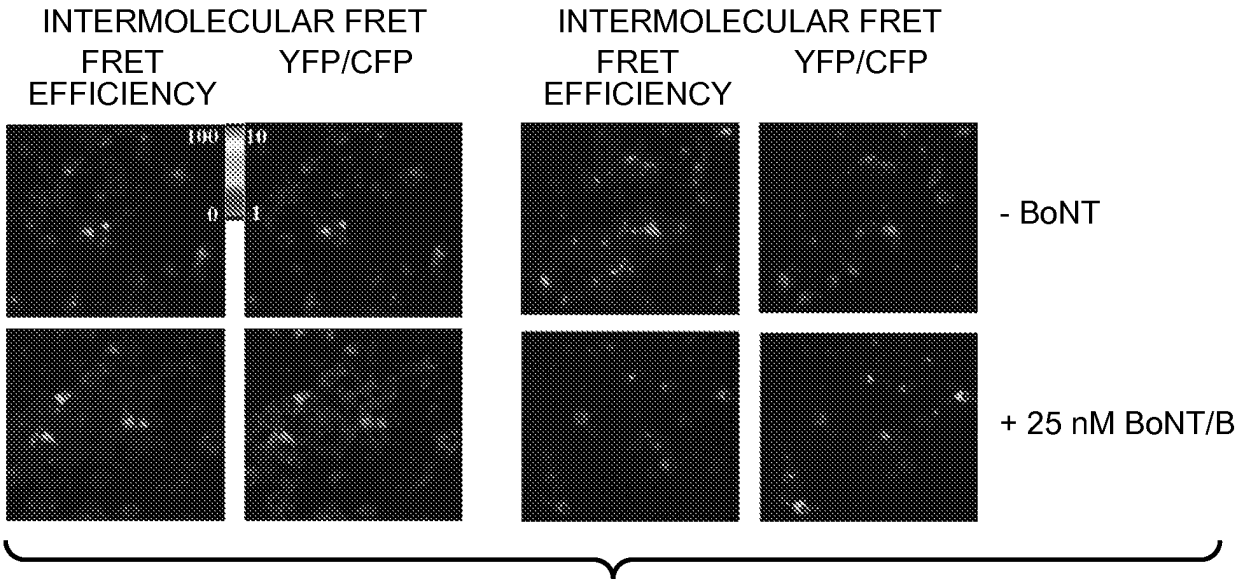
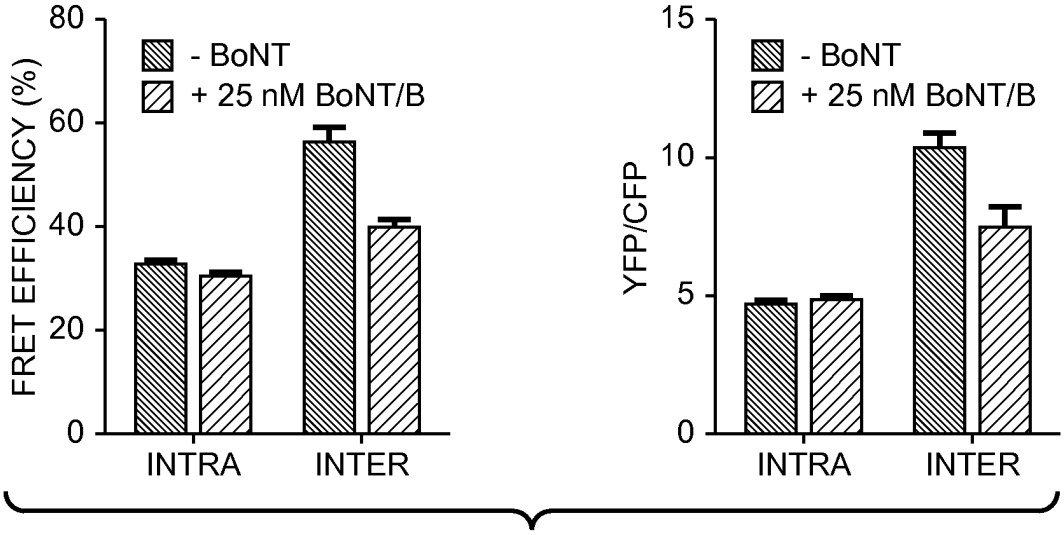

INTERMOLECULAR FRET
FRET EFFICIENCY
YFP/CFP
INTERMOLECULAR FRET
FRET EFFICIENCY
YFP/CFP
100
10
0
1
- BoNT
+ 25 nM BoNT/B

FIG. 4B


FRET EFFICIENCY (%)
80
60
40
20
0
- BoNT
+ 25 nM BoNT/B
INTRA
INTER
YFP/CFP
15
10
5
0
- BoNT
+ 25 nM BoNT/B
INTRA
INTER

REFERENCES CITED IN THE DESCRIPTION

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